Accepted for publication 2 April 2013. Published 30 July 2013.

Screening Selected Gulf Coast and Southeastern Forest Species for Susceptibility to *Phytophthora ramorum*

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Preuett, J. A., Collins, D. J., Luster, D., and Widmer, T. L. 2013. Screening selected Gulf Coast and southeastern forest species for susceptibility to *Phytophthora ramorum*. Online. Plant Health Progress doi:10.1094/PHP-2013-0730-01-RS.

Abstract

Phytophthora ramorum, the causal agent of sudden oak death, poses a threat to woody plants in the rest of the United States. Several plant species native to Gulf Coast and southeastern US forests were tested for reaction to *P. ramorum*, including eastern baccharis (*Baccharis halmifolia*), spicebush (*Lindera benzoin*), yaupon (*Ilex vomitoria*), southern magnolia (*Magnolia grandiflora*), sweetbay magnolia (*M. virginiana*), Virginia creeper (*Parthenocissus quinquefolia*), black willow (*Salix nigra*), and baldcypress (*Taxodium distichum*). The foliage of each species was inoculated with a zoospore suspension and placed in a dew chamber for 5 days. The average percentage of leaf area necrosis was 0.2, 4.9, 27.9, 32.1, 8.6, 1.5, 1.1, 0.2, and 5.0% for inoculated eastern baccharis, spicebush, yaupon, southern magnolia, sweetbay magnolia, Virginia creeper (Louisiana), Virginia creeper (Maryland), black willow, and baldcypress, respectively. Comparison of the percent necrotic leaf area between inoculated and non-inoculated plants showed significant differences ($P \leq 0.05$) for yaupon ($P = 0.0008$), southern magnolia ($P = 0.001$), and sweetbay magnolia ($P = 0.0009$). The other species did not show significant differences although infection was confirmed on spicebush, Virginia creeper, and baldcypress. This is a first report of yaupon, sweetbay magnolia, and baldcypress being hosts of *P. ramorum*.

Introduction

Since the discovery of *Phytophthora ramorum* in the western United States, numerous studies have examined the host range related specifically to this geographical location (8,15). Other studies (11,20,21) expanded the known host range to include oak species and understory plants that predominant or are native in the eastern US. However, species from the Gulf Coast region have not been examined adequately despite the region considered high risk for establishment of the pathogen (24).

There is concern that *P. ramorum* could spread throughout the US through the interstate movement of plant material (21). According to Chastagner et al. (2), infected nursery stock with *P. ramorum* has been found in Florida, Mississippi, Alabama, and Georgia. Suitable hosts and climatic conditions exists in the Gulf Coast region and eastern US that could support establishment of *P. ramorum* (9,10,24). If *P. ramorum* becomes established, the structure and biodiversity of the forests may be threatened. A recent study by Spaulding and Rieske (18) predicted a significant decline of red oaks in the Appalachian forests 10 years after a *P. ramorum* invasion. Similar results could be projected in the Gulf Coast region since there are numerous *Quercus* spp., in the coastal forest community (1), many of which are unknown to their susceptibility to *P. ramorum*. Forest understory species continue to drive the epidemic in California (4,8). There are numerous understory species in the Gulf Coast region that have also not been screened for susceptibility to *P. ramorum*. Therefore, it is

important to continue to expand the testing of plant species for their susceptibility to *P. ramorum* to help in improving the accuracy of risk models that can be useful in future management studies (20).

Plant Species Tested

This study was conducted in the Biosafety Level-3 containment greenhouse in Fort Detrick, MD, at the United States Department of Agriculture Agricultural Research Science Foreign Disease-Weed Science Research Unit (USDA-ARS FDWSRU). Selected woody understory vines, shrubs, and tree species were tested to determine their susceptibility to *Phytophthora ramorum* Werres, de Cock & Man in't Veld. Preliminary results of this study have been previously reported (14).

Plant material was chosen based upon their importance or ability to grow in the Gulf Coast region and availability of plant material for testing. Less than 1-year-old eastern baccharis shrubs (*Baccharis halmifolia* L.) were used belonging to the family *Asteraceae*. One-year-old spicebush plants [*Lindera benzoin* (L.) Blume], belonging to the family *Lauraceae* were propagated in the greenhouse. One-year-old yaupon shrubs (*Ilex vomitoria* Aiton), belonging to the family *Aquifoliaceae*, were obtained from Cleggs Nursery (Denham Springs, LA). Two-year-old southern magnolia trees (*Magnolia grandiflora* L.) were obtained from Forest Farm and 2- to 3-year-old sweetbay magnolia trees (*M. virginiana* L.) were obtained from Greenwood Nursery (McMinnville, TN), belonging to the family *Magnoliaceae*. Virginia creeper [*Parthenocissus quinquefolia* (L.) Planch], belonging in the family *Vitaceae*, was collected from the wild in Louisiana and Maryland and vegetatively propagated in the greenhouse. One-year-old black willow trees (*Salix nigra* Marshall), belonging in the family *Salicacea*e, were obtained from Forest Farm (Williams, OR). Two-year-old baldcypress trees [*Taxodium distichum* (L.) Rich.], belonging in the family *Cupressaceae*, were obtained from Nature Hills Nursery (Omaha, NE).

Culture and Inoculation Preparation

Cultures of *P. ramorum* were prepared based upon procedures by Mitchell and Kannwischer-Mitchell (12). *Phytophthora ramorum* isolate 5-C (NA1 lineage), originally collected from *Camellia sasanqua* Thunb. 'Bonanza' in California in 2003, was used in this study and maintained on 20% clarified V8 agar. This isolate was chosen because of its documented pathogenicity in other studies (17). Five plugs (5-mm diameter) containing mycelium of *P. ramorum* from the edge of an actively growing culture were transferred to Petri plates (100-mm diameter) containing approximately 20 ml of 20% clarified V8 broth. The plates were wrapped with plastic wrap and placed in an incubator at 20°C in the dark to allow for mycelia growth. After 4 days of incubation, the mycelia growing in the V8 broth were rinsed three times with 0.01 mM (2-Morpholinoethanesulfonic acid) buffer, pH 6.2 (herein referred to as MES buffer). The cultures were returned to the 20°C incubator overnight for formation of mature sporangia.

Zoospores were induced to release from the mature sporangia by placing the rinsed cultures at 4°C for 30 min and then allowing to sit at room temperature. Zoospore release was verified and filtered through a 53-micron screen. The zoospore concentration was calculated after the zoospore sample was diluted in MES buffer and vortexed to induce encystment. A hemacytometer was used to calculate the zoospore concentration per ml. Enough MES buffer was added to the zoospore suspension to prepare a final concentration of 50,000 zoospores/ml.

Plant Inoculations

Five plants of each species were selected based upon plant uniformity with similar plant age and various natural leaf growth stages. The foliage of four of the plants was sprayed evenly on both sides with the diluted zoospore suspension until run-off. One plant, used as a negative control, was sprayed only

with MES buffer. The plants were randomly placed in a dew chamber set at 20°C in 100% relative humidity for 5 days.

After 5 days, the plants were removed from the dew chamber. Except for yaupon, all leaves were detached from the plant and scanned abaxial-side down on an HP Scanjet 5500C model flatbed scanner (Hewlett-Packard, Palo Alto, CA). The resulting image was saved and analyzed using ASSESS 2.0 (American Phytopathological Society, St. Paul, MN) for necrotic leaf lesion area and total leaf area (cm^2). The percent necrotic leaf area per plant was calculated based upon this data. The experiment was conducted three times for each plant species.

Due to the abundant amount of leaves and small leaf size, yaupon foliar necrosis data was collected differently such that only a representative sample of the total plant was analyzed. While in the dew chamber after inoculation, some yaupon leaves defoliated (DL). These leaves were collected and pooled together after 5 days in the dew chamber. After removing the plants from the dew chamber, two or three branches (depending upon the number of leaves per branch) were randomly selected from each plant. The number of leaves on the selected branch were counted and set aside as a subsample (BL). The total leaves of each plant, including the defoliated and subsample leaves, were counted (TL). The percentage of leaves that represent the subsample in relation to the total leaves was calculated (%BL = BL/TL) for each plant. The number of collected defoliated leaves that need to be added back to the subsample (TDL) to give a complete representation of the total leaves on the subsample branches that included possible defoliated leaves was calculated (TDL = %BL*DL). The number of calculated TDL leaves was randomly selected from the DL leaf pool and added to the BL leaves, from which 120 leaves were randomly selected to be scanned and analyzed as described above for each plant.

To verify *P. ramorum* infection, a subsample of individual leaves or twigs displaying necrotic symptoms was selected from individual plants, surface-sterilized for 20 sec in 70% ethanol, rinsed three times in sterile distilled water, and placed on top of PARPH+V8 selective medium (6). Each plate was wrapped and placed for 1 week in the dark in a 20°C. *Phytophthora ramorum* infection was verified based on observations of characteristic hyphae, sporangia and chlamydospores (25).

Asymptomatic infection of Virginia creeper was also tested. Based on availability, four plants from Louisiana and two plants from Maryland were inoculated with *P. ramorum* zoospores as described above. After 5 days in the dew chamber, all asymptomatic leaves were removed, surface-sterilized in 70% ethanol for 20 sec, rinsed three times in sterile water and plated on PARPH+V8. The plates were examined after 1 week and rated positive if growth of *P. ramorum* was observed growing from the plated leaves.

Statistical Analyses

To avoid pseudo-replication, the mean percentage of necrotic leaf area for each plant was used as the subsample (5). Data failed the normality test based on the Shapiro-Wilk test ($P \geq 0.05$) and were transformed by arcsine transformation before analyses. The transformed data were analyzed by PROC GLM in SAS for Windows (Version 9.1, SAS Institute Inc., Cary, NC) and treatment means (non-inoculated versus inoculated plants) separated by least square means (LSM) for each individual species. *Phytophthora ramorum* was considered to be pathogenic on the individual species if $P \leq 0.05$ when compared to the non-inoculated control. In addition to analyzing species individually to their respective controls, inoculated Virginia creeper from Louisiana and Maryland and inoculated southern and sweetbay magnolia were compared to each other to gain an understanding of their relative susceptibility using GLM model using LSM.

Susceptibility to *Phytophthora ramorum*

Significant differences ($P \leq 0.05$) between the percent necrotic leaf area of inoculated plants and non-inoculated controls for each individual species were found for yaupon, southern magnolia, and sweetbay magnolia (Table 1). No significant differences were found for eastern baccharis, spicebush, Virginia creeper, black willow, and baldcypress. In this study *P. ramorum* was recovered from inoculated symptomatic plant material from spicebush, yaupon (Fig. 1B), both magnolia species (Fig. 1A), and baldcypress. The recovery of *P. ramorum* from infected spicebush and baldcypress leaves demonstrated that the two are hosts for *P. ramorum* and could serve as a potential inoculum source. Asymptomatic infections were recovered from inoculated plant material from both Virginia creeper populations.

Table 1. The average percent necrotic area per leaf of non-inoculated (control) and *Phytophthora ramorum*-inoculated tree, vine, and shrub species common to southeastern forests.

	Percent necrotic leaf area		**Statistical analysis**[x]
Forest plants	**Control**	**Inoculated**	**Pr > F**
Eastern baccharis	0.36	0.25	0.5696
Spicebush	1.16	4.92	0.2692
Yaupon	0.13	27.86	0.0008
Southern magnolia	0.56	32.06	0.001
Sweetbay magnolia	0.33	8.63	0.0009
Virginia creeper (Louisiana)	3.1	1.48	0.4105
Virginia creeper (Maryland)	1.08	1.14	0.6494
Black willow	0.35	0.15	0.3013
Baldcypress	4.24	4.95	0.4446
Magnolia species cross comparison[y]	–	–	**<0.0001**
Virginia creeper populations cross comparison[z]	–	–	**0.5364**

[x] Comparsion of non-inoculated and *P. ramorum*-inoculated plants using PROC GLM to determine significance ($P < 0.05$).

[y] Comparison of *P. ramorum*-inoculated southern magnolia and sweetbay magnolia.

[z] Comparison of *P. ramorum*-inoculated Virginia creeper genotypes from Louisiana and Maryland.

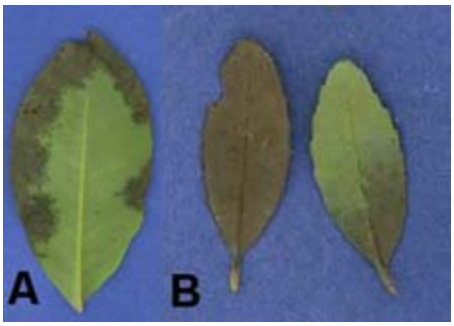

Fig. 1. Leaf necrosis symptoms of sweetbay magnolia (**A**) and yaupon (**B**) after artificial inoculation with *Phytophthora ramorum* zoospores.

Eastern baccharis and black willow results. Based on the results of this study (Table 1), eastern baccharis and black willow appear to be resistant to *P. ramorum* and should not be considered potential hosts. These two were originally selected because of their prominence in the Gulf Coast region that would have been a concern if found to be infected.

Spicebush results. Infection of spicebush in this study confirms other studies that have shown similar necrosis of this host (11,20). Infection of spicebush is significant because it is a very common understory plant in eastern US forests and any infection or sporulation may be sufficient to drive an epidemic. Understory plant species play an important role in the epidemiology of *P. ramorum* (3,20). It is has been demonstrated that the understory species, *Umbellularia californica* (California bay laurel), is responsible for maintaining the epidemic in California (3).

Yaupon results. The percentage of necrosis of inoculated yaupon leaves (Table 1) was found to be significantly higher than the non-inoculated controls and had a relatively high percentage of necrotic leaf area (>27%). *Ilex vomitoria* has not been reported previously as a host to *P. ramorum* although other *Ilex* spp. are known associated hosts (23). Inoculated yaupon leaves naturally defoliated as a result of infection. Although in this study yaupon leaves were not investigated as to whether they contained *P. ramorum* propagules, the defoliated leaves could be a secondary inoculum source in nature if they do contain chlamydospores. According to Shishkoff (17), *P. ramorum* propagules can survive in the soil from fallen plant material and could serve as an inoculum source. Yaupon is a commonly found landscape plant in the southeastern US and sold in retail nurseries. Infected nursery stock could facilitate the spread of *P. ramorum* throughout the region and to yaupon growing naturally in the wild. Yaupon is a plant that needs to be investigated further.

Southern and sweetbay magnolia results. This is the first confirmation of the susceptibility of sweetbay magnolia to *P. ramorum*. A hybrid cross between *M. tripetala* and *M. virginiana* is on the USDA/APHIS list of plants associated with *P. ramorum*, but no previous studies have examined *M. virginiana*. Southern magnolia has been recorded as an associated host of the organism (23). Additional statistical analysis to compare the two magnolias showed that the percentage of necrosis was significantly different between the two species (Table 1). This difference demonstrates that there might be some degree of tolerance in *Magnolia* spp. This difference among species within a genus is not unusual as has been demonstrated previously in other studies inoculating whole plants (16,20,22,26).

Virginia creeper asymptomatic infection results. The inoculated Virginia creeper from Louisiana and Maryland showed less than 1.5% necrosis (Table 1). However, when leaflets from inoculated Virginia creeper were placed on the selective medium for verification of *P. ramorum* infection, mycelium was observed growing from the asymptomatic leaves. A follow-up study estimated that 10 and 13.6% of the asymptomatic leaves from the Louisiana and Maryland populations, respectively, were infected. Virginia creeper is a vine that could reach into the high canopies of susceptible tree species facilitating the spread of *P. ramorum*. Further study is needed on Virginia creeper to better understand its potential role in the Gulf Coast region as a potential inoculum source for *P. ramorum*.

Baldcypress results. Baldcypress is not listed on the APHIS confirmed or associated host lists (23) and has not been tested previously for its susceptibility to *P. ramorum*. Baldcypress, which can grow and survive in prolonged and frequent flooded conditions fatal to most species, is an important canopy species found in the southeastern US (27). The natural flooded wetland areas in deep water swamps and bottomland forests where this species is found in standing water could serve as an avenue to spread *P. ramorum* should a plant get infected. *Phytophthora ramorum* has been demonstrated to disseminate via local waterways (19). Although the exact source of propagules in waterways has not been demonstrated conclusively, it is believed that fallen infected plant material may play a role (13). Based on the ecological importance of baldcypress, increased knowledge of its reaction to *P. ramorum* and its ability to support

sporulation can determine the potential risk to the Gulf Coast region if *P. ramorum* becomes established.

Conclusion

This is a first report of the susceptibility of yaupon, sweetbay magnolia, Virginia creeper, and baldcypress to *P. ramorum* infection under artificial inoculation. The most significant findings of this study were the high susceptibility of yaupon and the asymptomatic infection of Virginia creeper. Both of these species are prevalent throughout the Gulf Coast region and could play a significant role in the epidemiology of this disease should *P. ramorum* become established in the ecosystem.

Although these studies were done under laboratory conditions, they still give valuable information on host plants that could perpetuate the pathogen's life cycle. With the knowledge that understory forest species play a vital role as an inoculum source, it is important to continue to test new species to improve the accuracy of risk analyses (20). If *P. ramorum* becomes established, the structure and biodiversity of North America native forests may be threatened (7). Less documentation exists about the susceptibility of eastern native plant species to *P. ramorum* and the sporulation potential compared to west coast plant species (11,20) and no previous studies specifically to the Gulf Coast region even though it is a high risk area (24).

Acknowledgments and Disclaimers

The authors wish to thank Dr. Nina Shishkoff, Mr. Stephen Dodge, and Mr. Tommy Frazier for their excellent technical assistance and providing plant material. Funding for the research was provided by USDA NIFA 1890 Capacity Building Grant 2010-38821-21539.

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